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Identification and Characterization of the *cps* Locus of *Streptococcus suis* Serotype 2: the Capsule Protects against Phagocytosis and Is an Important Virulence Factor

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To study the role of the capsule of *Streptococcus suis* serotype 2 in virulence, we generated two isogenic mutants disturbed in capsule production. For that purpose, we first cloned and characterized a major part of the capsular polysaccharide biosynthesis (*cps*) locus of *S. suis* serotype 2. Based on the established sequence, 14 open reading frames (ORFs), designated Orf2Z, Orf2Y, Orf2X, and Cps2A to Cps2K, were identified. Twelve ORFs belonged to a single transcriptional unit. The gene products of 11 of these ORFs showed similarity to proteins involved in polysaccharide biosynthesis of other gram-positive microorganisms. Nonencapsulated isogenic mutants were generated in the *cps2B* and *cps2EF* genes by insertional mutagenesis. In contrast to the wild-type *S. suis* serotype 2 strain, the nonencapsulated strains were highly sensitive to ingestion by porcine alveolar lung macrophages in vitro. More importantly, the nonencapsulated mutant strains were completely avirulent in young germfree pigs after intranasal inoculation. These observations indicate that the capsule of *S. suis* serotype 2 plays an essential role in the pathogenesis of *S. suis* serotype 2 infections.

Streptococcus suis is an important cause of meningitis, septicemia, arthritis, and sudden death in young pigs (4, 38). It can, however, also cause human meningitis (1). *S. suis* strains are identified by their morphological, biochemical, and serological characteristics. Serological classification is based on the presence of specific epitopes on its polysaccharidic capsule. So far, 35 different serotypes have been described (8, 13). Strains of *S. suis* can differ in virulence. Some serotypes are more frequently isolated from diseased pigs than others, suggesting that differences in virulence are associated with differences in capsular polysaccharides. In Europe, *S. suis* serotype 2 is the type most frequently isolated from diseased pigs, followed by serotypes 9 and 1. The idea that the capsule of *S. suis* serotype 2 plays a role in the pathogenesis was supported by the observation of reduced virulence for transposon mutants of *S. suis* impaired in capsule production (3). Moreover, it is well known that the levels of virulence of *S. suis* strains within a single serotype can differ greatly (37, 39). A number of strains of *S. suis* serotypes 1 and 2 have been shown to be highly virulent in pigs, whereas other strains of serotypes 1 and 2 are completely avirulent (33, 37, 39). Both the virulent and avirulent strains of either serotype seem to be fully encapsulated. This suggests that there is only a minor contribution of the capsule to the virulence of *S. suis*. Indeed, various bacterial components, such as extracellular and cell membrane-associated proteins, fimbriae, hemagglutinins, and hemolysin, have been suggested as virulence factors (7, 9, 10, 14, 15, 39, 41). However, the precise role of these protein components in the pathogenesis of the disease has not been established (29).

To provide conclusive evidence with regard to the role and contribution of the capsule of *S. suis* in determining virulence, we identified and characterized a major part of the DNA region encoding the proteins necessary for capsule synthesis. In addition, we generated isogenic mutants in two different capsular genes. Both isogenic mutants were found to be resistant to phagocytosis by alveolar lung macrophages in vitro. In addition, the nonencapsulated mutants were completely avirulent in young germfree pigs.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. suis* strains were grown in Todd-Hewitt broth (code CM189; Oxoid) and plated on Columbia agar blood base (code CM331; Oxoid) containing 6% (vol/vol) horse blood. *Escherichia coli* strains were grown in Luria broth (23) and plated on Luria broth containing 1.5% (wt/vol) agar. If required, antibiotics were added to the plates at the following concentrations: spectinomycin, 100 μg/ml for *S. suis* and 50 μg/ml for *E. coli*; ampicillin, 50 μg/ml for *E. coli*.

Serotyping. The *S. suis* strains were serotyped by the slide agglutination test with serotype-specific antibodies (36).

Selection of genes encoding exported proteins. Chromosomal DNA of *S. suis* serotype 2 was digested with *Alu*I. The 300- to 500-bp fragments were ligated to *Sma*I-digested pPHOS2. Ligation mixtures were transformed to PhoA$^-$ *E. coli* CC118. Transformants were plated on Luria broth agar plates supplemented with 5-bromo-4-chloro-3-indolylphosphate (BCIP) (50 μg/ml) (Boehringer, Mannheim, Germany). Blue colonies were purified on fresh Luria broth/BCIP plates to verify the blue phenotype.

DNA techniques and sequence analysis. Routine DNA manipulations were performed as described by Sambrook et al. (28). DNA sequences were determined on a 373A DNA Sequencing System (Applied Biosystems, Warrington, Great Britain). Samples were prepared by use of an ABI/PRISM dye terminator cycle sequencing ready reaction kit (Applied Biosystems). Sequencing data were assembled and analyzed by using the MacMollyTetra software package. Custom-made sequencing primers were purchased from Life Technologies. The BLAST software package was used to search for protein sequences homologous to the deduced amino acid sequences in the GenBank/EMBL databases.

Construction of gene-specific knock out mutants. To construct the mutant strains 10cpsΔB and 10cpsΔEF, we electrotransformed pathogenic strain 10 (37, 41) of *S. suis* serotype 2 with pCPS11 and pCPS28, respectively. In these plasmids the *cpsB* and *cpsEF* genes are inactivated by the insertion of a spectinomycin resistance gene. To create pCPS11, the internal 400-bp *Pst*I-*Bam*HI fragment of the *cpsB* gene in pCPS7 was replaced by the 1,200-bp *Pst*I-*Bam*HI fragment from pIC-spc, containing the spectinomycin resistance gene. To construct pCPS28 we have used pIC20R. Into this plasmid we inserted the *Kpn*I-*Sal*I fragment from pCPS17 (resulting in pCPS25) and the *Xba*I-*Cla*I fragment from pCPS20 (resulting in pCPS27). pCPS27 was digested with *Pst*I and *Xho*I and ligated to the

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. coli		
CC118	PhoA−	21
XL2 blue		Stratagene
S. suis		
10	Virulent serotype 2 strain	40
10cpsB	Isogenic cpsB mutant of strain 10	This work
10cpsEF	Isogenic cpsEF mutant of strain 10	This work
Plasmids		
pKUN19	Replication functions pUC, Amp^r	19
pGEM7Zf(+)	Replication functions pUC, Amp^r	Promega Corp.
pIC19R	Replication functions pUC, Amp^r	22
pIC20R	Replication functions pUC, Amp^r	22
pIC-spc	pIC19R containing Spc^r gene of pDL282	Lab collection
pDL282	Replication functions of pBR322 and pVT736-1, Amp^r, Spc^r	31
pPHOS2	pIC-spc containing the truncated *phoA* gene of pPHO7 as a *Pst*I-*Bam*HI fragment	This work
pPHO7	Contains truncated *phoA* gene	12
pPHOS7	pPHOS2 containing chromosomal *S. suis* DNA	This work
pCPS6	pKUN19 containing 6-kb *Hin*dIII fragment of *cps* operon	This work (Fig. 1)
pCPS7	pKUN19 containing 3.5-kb *Eco*RI-*Hin*dIII fragment of *cps* operon	This work (Fig. 1)
pCPS11	pCPS7 in which 0.4-kb *Pst*I-*Bam*HI fragment of *cpsB* gene is replaced by Spc^r gene of pIC-spc	This work (Fig. 1)
pCPS17	pKUN19 containing 3.1-kb *Kpn*I fragment of *cps* operon	This work (Fig. 1)
pCPS18	pKUN19 containing 1.8-kb *Sna*BI fragment of *cps* operon	This work (Fig. 1)
pCPS20	pKUN19 containing 3.3-kb *Xba*I-*Hin*dIII fragment of *cps* operon	This work (Fig. 1)
pCPS23	pGEM7Zf(+) containing 1.5-kb *Mlu*I fragment of *cps* operon	This work (Fig. 1)
pCPS25	pIC20R containing 2.5-kb *Kpn*I-*Sal*I fragment of pCPS17	This work (Fig. 1)
pCPS26	pKUN19 containing 3.0-kb *Hin*dIII fragment of *cps* operon	This work (Fig. 1)
pCPS27	pCPS25 containing 2.3-kb *Xba*I (blunt)-*Cla*I fragment of pCPS20	This work (Fig. 1)
pCPS28	pCPS27 containing the 1.2-kb *Pst*I-*Xho*I Spc^r gene of pIC-spc	This work (Fig. 1)

[a] Amp^r, ampicillin resistant; Spc^r, spectinomycin resistant; and cps, capsular polysaccharide.

1,200-bp *Pst*I-*Xho*I fragment, containing the spectinomycin resistance gene of pIC-spc. The electrotransformation to *S. suis* was carried out as described before (30).

Southern blotting and hybridization. Chromosomal DNA was isolated as described by Sambrook et al. (28). DNA fragments were separated on 0.8% agarose gels and transferred to Zeta-Probe GT membranes (Bio-Rad) as described by Sambrook et al. (28). DNA probes were labelled with [α-^{32}P]dCTP (3,000 Ci mmol^{-1}; Amersham) by use of a random primed labelling kit (Boehringer). The DNA on the blots was hybridized at 65°C with the appropriate DNA probes as recommended by the supplier of the Zeta-Probe membranes. After hybridization, the membranes were washed twice with a solution of 40 mM sodium phosphate (pH 7.2), 1 mM EDTA, and 5% sodium dodecyl sulfate for 30 min at 65°C and twice with a solution of 40 mM sodium phosphate (pH 7.2), 1 mM EDTA, and 1% sodium dodecyl sulfate for 30 min at 65°C.

Electron microscopy. Bacteria were prepared for electron microscopy as described by Wagenaar et al. (42). Shortly, bacteria were mixed with agarose MP (Boehringer) of 37°C to a concentration of 0.7%. The mixture was immediately cooled on ice. Upon gelling, samples were cut into 1- to 1.5-mm-thick slices and incubated in a fixative containing 0.8% glutaraldehyde and 0.8% osmium tetroxide. Subsequently, the samples were fixed and stained with uranyl acetate by microwave stimulation, dehydrated, and embedded in eponaraldite resin. Ultrathin sections were counterstained with lead citrate and examined with a Philips CM 10 electron microscope at 80 kV. In addition, we used the polycationic ferritin method as described by Quessy et al. (26).

Phagocytosis assay. Porcine alveolar macrophages (AM) were obtained from the lungs of specific-pathogen-free (SPF) pigs. Lung lavage samples were collected as described by van Leengoed et al. (35). Cells were suspended in Eagle's minimal essential medium (EMEM) containing 6% (vol/vol) SPF pig serum and adjusted to 10^7 cells per ml. Phagocytosis assays were performed as described by Leij et al. (20). Briefly, 10^7 *S. suis* cells were incubated with 6% SPF pig serum for 30 min at 37°C in a head-over-head rotor at 6 rpm, to opsonize the cells. We combined 10^7 AM and 10^7 opsonized *S. suis* cells and incubated them at 37°C under continuous rotation at 6 rpm. At 0, 30, 60, and 90 min, 1-ml samples were collected and mixed with 4 ml of ice-cold EMEM to stop phagocytosis. Phagocytes were removed by centrifugation for 4 min at 110 × *g* and 4°C. The number of CFU in the supernatants was determined by plating. Control experiments were carried out simultaneously by combining 10^7 opsonized *S. suis* cells with EMEM without AM.

Killing assays. Killing assays were performed as described by Leij et al. (20). AM (10^7/ml) and opsonized *S. suis* cells (10^7/ml) were mixed 1:1 and incubated for 10 min at 37°C under continuous rotation at 6 rpm. Ice-cold EMEM was added to stop further phagocytosis and killing. To remove extracellular *S. suis* cells, phagocytes were washed twice (4 min, 110 × *g*, 4°C) and resuspended in 5 ml of EMEM containing 6% SPF pig serum. The resuspended AM were incubated at 37°C under rotation at 6 rpm. After 0, 15, 30, 60, and 90 min, samples were collected and mixed with ice-cold EMEM to stop further killing. The samples were centrifuged for 4 min at 110 × *g* at 4°C, and the phagocytic cells were lysed in EMEM containing 1% saponin for 20 min at room temperature. The number of CFU in the suspensions was determined by plating.

Experimental infections. Germfree pigs, crossbreeds of Great Yorkshire and Dutch Landrace, were obtained from sows by cesarean sections. The surgery was performed in sterile flexible film isolators. Pigs were allotted to groups, each consisting of 4 pigs, and were housed in sterile stainless steel incubators. Housing conditions and feeding regimens were as described before (37, 41). Pigs were inoculated intranasally with *S. suis* serotype 2 as described before (37, 41). To predispose the pigs to infection with *S. suis*, 5-day-old pigs were inoculated intranasally with about 10^7 CFU of *Bordetella bronchiseptica* 92932. Two days later the pigs were inoculated intranasally with *S. suis* serotype 2 (10^6 CFU). Pigs were monitored twice daily for clinical signs of disease, such as fever, nervous signs, and lameness. Blood samples were collected three times a week from each pig. Leucocytes were counted with a cell counter. To monitor infection with *S. suis* and *B. bronchiseptica* and to check for absence of contaminants, we collected swabs of the nasopharynx and the feces daily. The swabs were plated directly onto Columbia agar containing 6% horse blood. After the pigs were killed, they were examined for pathological changes. Tissue specimens from the central nervous system (CNS), serosae, and joints were examined bacteriologically and histologically as described before (37, 41). Colonization of the serosae was scored positively when *S. suis* was isolated from the pericardium, thoracic pleura, or peritoneum. Colonization of the joints was scored positively when *S. suis* was isolated from one or more joints (12 joints per animal were scored).

Nucleotide sequence accession number. The nucleotide sequence data have been submitted to GenBank under accession no. AF118389.

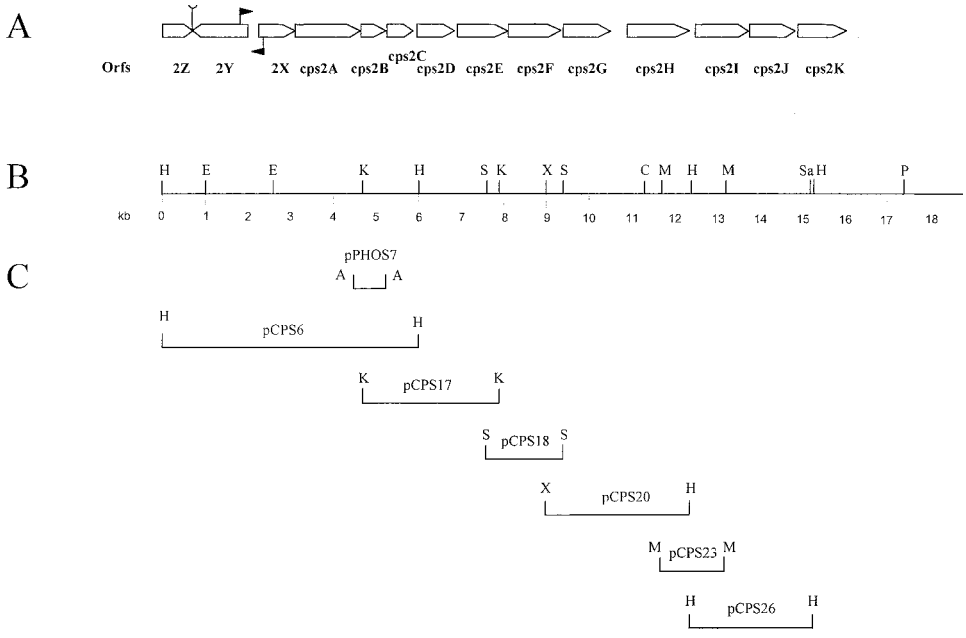

FIG. 1. Organization of the *cps2* gene cluster of *S. suis* type 2. (A) Genetic map of the *cps2* gene cluster. The open arrows represent potential ORFs. Gene designations are indicated below the ORFs. The closed arrows indicate the position of the potential promoter sequences. | indicates the position of the potential transcription regulator sequence. (B) Physical map of the *cps2* locus. (C) The DNA fragments cloned in the various plasmids. Restriction sites are as follows: A, *Alu*I; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; P, *Pst*I; S, *Sna*BI; Sa, *Sac*I; X, *Xba*I.

RESULTS

Identification and isolation of capsule-encoding DNA. Initially, a part of the capsular locus of *S. suis* serotype 2 was isolated in an attempt to identify secreted proteins by genetic means (12, 25). For this purpose chromosomal DNA of *S. suis* serotype 2 was cloned in *E. coli* in front of a 5′-truncated alkaline phosphatase gene. To do this, we made use of the vector pPHOS2 (Table 1), which contained the truncated alkaline phosphatase gene of pPHO7 (12) as well as a spectinomycin resistance gene (31). A number of *E. coli* clones displayed a dark blue phenotype when plated on media containing BCIP, indicating that the cloned fragment contained a promoter, a translational start site, and a signal sequence. The deduced amino acid sequence of one of the cloned fragments (on plasmid pPHOS7) showed a high similarity (37% identity) to a protein (Cps14C) involved in capsular synthesis of *Streptococcus pneumoniae* (18). This strongly suggested that pPHOS7 contained a part of the corresponding *cps* gene of *S. suis* serotype 2. Subsequently, the insert of pPHOS7 (Fig. 1C) was used as a probe to identify chromosomal DNA fragments containing flanking *cps* genes. A 6-kb *Hin*dIII fragment was identified and cloned in pKUN19. This yielded clone pCPS6 (Fig. 1C). Sequence analysis of the insert revealed that pCPS6 contained the 5′ end of the *cps* locus. Sequences of the 3′ end of pCPS6 were, in turn, used to identify a chromosomal fragment containing *cps* sequences located further downstream. This fragment was also cloned in pKUN19, resulting in pCPS17. Using a similar approach, we subsequently isolated the plasmids pCPS18, pCPS20, pCPS23, and pCPS26 containing downstream *cps* sequences (Fig. 1C).

Analysis of the *cps* operon. The complete nucleotide sequences of the cloned fragments were determined. Examination of the compiled sequence revealed the presence of 14 potential open reading frames (ORFs), which were designated Orf2Z, Orf2Y, Orf2X, and Cps2A through Cps2K (Fig. 1A). Orf2Z, located at the 5′ end of the sequence, was incomplete. Compared to the other ORFs, Orf2Y is expressed in the opposite orientation. Two potential promoter sequences were identified. One was located 313 bp (positions 1885 to 1865 and 1884 and 1889) upstream of Orf2X. The other was located 68 bp upstream of Orf2Y (positions 2241 to 2236 and 2216 to 2211). Between Orf2Y and Orf2Z the sequence contained a potential stem-loop structure, which could act as a transcription terminator. Each ORF is preceded by a ribosome-binding site, and the majority of the ORFs are very closely linked. The only significant intergenic gap was that found between Cps2G and Cps2H (389 nucleotides). No obvious promoter sequences or potential stem-loop structures were found in this region. This suggests that Orf2X and Cps2A through Cps2K are part of a single transcriptional unit.

A list of all ORFs with their properties is shown in Table 2. Orf2Z showed similarity to the YitS protein of *Bacillus subtilis*, a protein with an unknown function. Orf2Y showed homology to the YcxD protein of *B. subtilis* (43), which is supposed to be a regulatory protein. Orf2X showed homology with the hypothetical YAAA proteins with unknown function of *Haemophilus influenzae* and *E. coli*. The products of the *cps2A*, *cps2B*, *cps2C*, and *cps2D* genes showed significant homologies with the CpsA, CpsC, CpsD, and CpsB proteins of several streptococci (Table 2), suggesting similar functions for these proteins. Hence, Cps2A may have a role in the regulation of the capsular polysaccharide synthesis, Cps2B and Cps2C could be involved in the chain length determination of the type 2 capsule, and Cps2C could play an additional role in the export of the polysaccharide. Cps2D is homologous to Cps proteins of streptococci involved in the polysaccharide or exopolysaccharide synthesis, but it is without a known specific function (18). The proteins encoded by the *cps2E*, *cps2F*, *cps2G*, *cps2H*, *cps2J*, and *cps2K* genes showed homology to proteins with glycosyltransferase activities of several streptococci (5, 16, 17, 18, 32), suggesting that these proteins are involved in the biosynthesis of the type 2 oligosaccharide subunit. The protein encoded by the

TABLE 2. Properties of ORFs in the *cps* locus of *S. suis* serotype 2 and similarities to gene products of other bacteria

ORF	Nucleotide positions in sequence	No. of amino acids	Proposed function of gene product[a]	Bacterial strain(s) with similar gene product (% identity)	Reference or accession no.
Orf2Z	1–719	240	Unknown	*B. subtilis* YitS (26)	Y09478
Orf2Y	2079–822	419	Transcription regulation	*B. subtilis* YcxD (39)	43
Orf2X	2202–2934	244	Unknown	*H. influenzae* YAAA (24)	P43908
Cps2A	3041–4484	481	Regulation	*S. pneumoniae* Cps19fA (58)	11, 24
Cps2B	4504–5191	229	Chain length determination	*S. pneumoniae* type 3 Orf1 (58)	2
Cps2C	5203–5878	225	Chain length determination/export	*S. pneumoniae* Cps23fD (63)	5
Cps2D	5919–6648	243	Unknown	*S. pneumoniae* CpsB (62)	5
Cps2E	6675–8052	459	Glycosyltransferase	*S. pneumoniae* Cps14E (56)	16, 18
Cps2F	8089–9256	389	Glycosyltransferase	*S. pneumoniae* Cps23fT (72)	5
Cps2G	9262–10417	385	Glycosyltransferase	*S. thermophilus* EpsF (25)	32
Cps2H	10808–12176	457	Glycosyltransferase	*S. mutans* RGPEC[b] (29)	D1033055
Cps2I	12213–13443	410	Capsular polysaccharide polymerase	*S. pneumoniae* Cps23fI (48)	5
Cps2J	13583–14579	332	Glycosyltransferase	*S. pneumoniae* Cps14J (31)	17
				S. pneumoniae Cps 14I (27)	17
Cps2K	14574–15401	276	Glycosyltransferase	*S. pneumoniae* Cps14J (40)	17

[a] Predicted by sequence similarity.
[b] Similarity refers to the amino-terminal part of the gene product.

cps2I gene showed homology to a protein of *S. pneumoniae* with potential polysaccharide polymerase activity (5).

Construction of mutants impaired in capsule synthesis. To evaluate the role of the capsule of *S. suis* serotype 2 in virulence, we constructed two isogenic mutants in which capsule production was disturbed. To construct mutants 10cpsΔB and 10cpsΔEF, the plasmids pCPS11 and pCPS28 were used. pCPS11 and pCPS28 were electrotransformed into strain 10 of *S. suis* serotype 2, and spectinomycin-resistant colonies were selected. Southern blotting and hybridization experiments were used to select double crossover integration events (data not shown).

To test whether the capsular structure of the mutant strains 10cpsΔB and 10cpsΔEF was disturbed, we used a slide agglutination test (36). The parent strain, strain 10, of *S. suis* serotype 2 agglutinated only in type 2-specific serum. The mutant strains, however, agglutinated in sera specific for all *S. suis* strains, but they also agglutinated in the absence of serotype-specific serum. This indicated that in the mutant strains the capsular structure was disturbed. To confirm this, thin sections of wild-type and mutant strains were compared by electron microscopy. Compared to the wild-type strain (Fig. 2A), the amount of capsule produced by the mutant strains was greatly reduced (Fig. 2B and C). No capsular material could be detected on the surfaces of the mutant strains. Similar results were obtained after the polycationic ferritin method was used (results not shown).

Capsular mutants are sensitive to phagocytosis and killing by AM. The capsular mutants and the parent strain were tested for the ability to resist phagocytosis by AM in the presence of porcine SPF serum. As shown in Fig. 3A, the wild-type strain (strain 10) is resistant to phagocytosis under the in vitro conditions used (Fig. 3A). In contrast, both mutant strains were efficiently ingested by the macrophages (Fig. 3A). After 90 min, more than 99.7% (strain 10cpsΔB) and 99.8% (strain 10cpsΔEF) of the mutants were ingested by the macrophages. Moreover, as shown in Fig. 3B, the ingested strains were efficiently killed by the macrophages. From 90 to 98% of all ingested cells were killed within 90 min. No differences in killing efficiency could be observed between wild-type and mutant strains. Similar results were obtained after polymorphonuclear leukocytes were used (results not shown). These data indicate that the capsule of *S. suis* serotype 2 efficiently protects the bacterium from uptake by macrophages in vitro.

Capsular mutants are avirulent in germfree piglets. The virulence properties of the wild-type and mutant strains were tested by experimental infection of newborn germfree pigs (37, 41). Table 3 shows that specific and nonspecific signs of disease could be observed in all pigs inoculated with the wild-type strain. All pigs inoculated with the wild-type strain died during the course of the experiment or were killed because of serious illness or nervous disorders (Table 3). In contrast, the pigs inoculated with strains 10cpsΔB or 10cpsΔEF showed no specific signs of disease and all of these pigs survived until the end of the experiment. Moreover, we observed significant differences in the fever index and in the leukocyte index between pigs inoculated with wild-type and mutant strains (Table 3). *S. suis* strains and *B. bronchiseptica* could be isolated from the nasopharyngeal and fecal swab samples of all pigs from 1 day postinfection until the end of the experiment. Postmortem, the wild-type strain could frequently be isolated from the CNS, kidney, heart, liver, spleen, serosae, joints, and tonsils. Mutant strains could be recovered from the tonsils but were never recovered from the kidney, liver, or spleen. Interestingly, small numbers of the mutant strains could be isolated from the CNS, the serosae, the joints, the lungs, and the heart. Agglutination tests and Southern blot analyses showed that these mutant strains had the unencapsulated phenotype and genotype (results not shown). Taken together, these data demonstrate that mutant *S. suis* strains impaired in capsule production are avirulent in young germfree pigs.

DISCUSSION

In the present paper we describe the identification and the molecular characterization of a 16-kb DNA fragment containing a major part of the genetic determinant involved in the capsular polysaccharide biosynthesis of *S. suis* serotype 2. To study the role of the capsule in resistance to phagocytosis and in virulence, we constructed two isogenic mutants in which capsule synthesis was disturbed. In 10cpsΔB, the *cps2B* gene was disturbed by the insertion of an antibiotic resistance gene, whereas in 10cpsΔEF parts of the *cps2E* and *cps2F* genes were replaced by an antibiotic resistance gene. By electron micro-

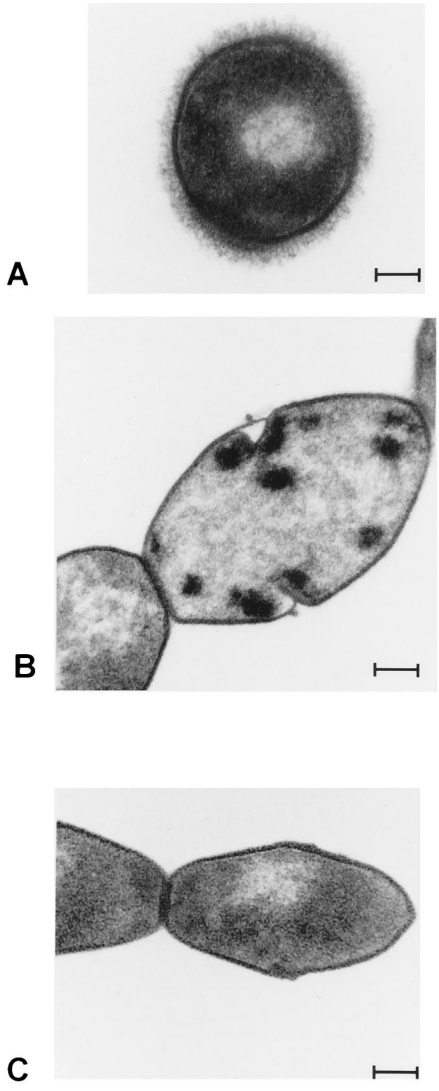

FIG. 2. Transmission electron micrographs of thin sections of various *S. suis* strains. Panels: A, wild-type strain, strain 10; B, mutant strain 10cpsΔB; and C, mutant strain 10cpsΔEF. Bar = 100 nm.

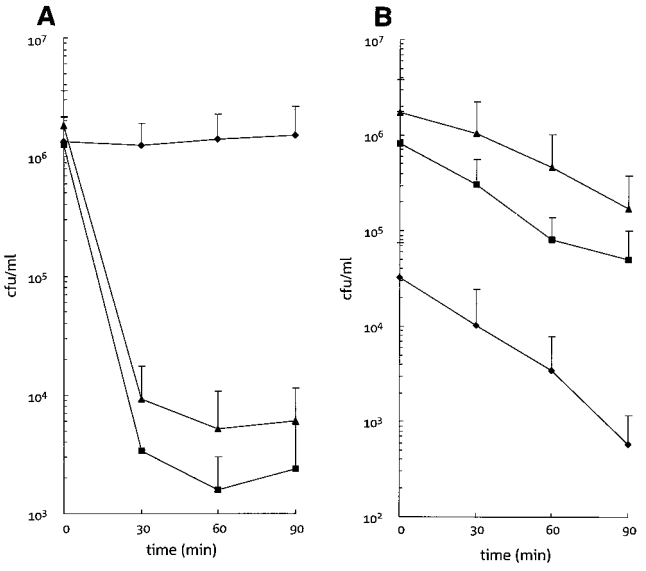

FIG. 3. (A) Kinetics of phagocytosis of wild-type and mutant *S. suis* strains by porcine AM. Average data of several experiments are presented. Bars are standard deviations. (B) Kinetics of intracellular killing of wild-type and mutant *S. suis* strains by porcine AM. Average data of several experiments are represented. Bars are standard deviations. ◆, wild-type strain, strain 10; ▲, mutant strain 10cpsΔB; ■, mutant strain 10cpsΔEF.

scopical analysis both mutant strains were found to be completely unencapsulated. Although this finding confirms that the *cpsB* and *cpsEF* genes are involved in capsular synthesis, this finding does not give any clues to the function of these proteins, since they form part of an operon structure and polar effects on the expression of downstream genes cannot be excluded. The behavior of the mutants in the in vitro phagocytosis and killing assays clearly showed that the capsular polysaccharide of *S. suis* serotype 2 is a surface component with antiphagocytic activity. Wild-type encapsulated bacteria were ingested by phagocytes at a very low frequency, whereas the mutant unencapsulated bacteria were efficiently ingested by porcine macrophages. Once ingested, wild-type and mutant strains seemed to be killed with the same relative efficiency. This suggests that the loss of capsular material is associated with loss of capacity to resist uptake by macrophages. This loss of resistance to in vitro phagocytosis was associated with an almost complete attenuation of the virulence of the mutant strains in germfree pigs. All pigs inoculated with the mutant strains survived the experiment and did not show any specific clinical signs of disease. Only some nonspecific clinical signs of disease could be observed. Moreover, small numbers of mutant bacteria could be reisolated from the pigs. This supports the idea that, as in other pathogenic streptococci, the capsule of *S. suis* acts as an important virulence factor. Our data obtained with the isogenic mutants are in agreement with the data recently reported by Charland et al. (3). They reported that transposon mutants, which are impaired in capsule production, showed reduced virulence in pigs and mice (3). However, to construct these transposon mutants, the authors used the serotype 2 reference strain S735. We previously showed that strain S735 is only weakly virulent for young pigs (40). Moreover, since the insertion sites of the transposon in the mutants were not determined, it could not be concluded that the observed reduction in virulence was a direct consequence of impaired capsule synthesis.

Initially a part of the *cps2B* gene was cloned by screening for signal sequences. The hydrophobicity profile of the clone showed that the N-terminal part of the sequence resembled the characteristics of a typical signal peptide: a short N-terminal region is followed by a hydrophobic region of 38 amino acids. Apparently, this region was able to translocate alkaline phosphatase across the cellular membrane in *E. coli*. The hydrophobicity plot of the corresponding Cps14C protein of *S. pneumoniae* showed two hydrophobic segments, one each at its N and C termini, and a hydrophilic domain in the central part (18). The cellular location of this protein is unknown. The region homologous to the second hydrophobic domain was not cloned in pPHOS7.

The cloned and sequenced region described here contained 14 ORFs. At least 12 of these ORFs belong to a single transcriptional unit, suggesting a coordinated control of the expression of these genes. Based on sequence similarities we could assign putative functions to most of the gene products. We thereby identified gene products involved in regulation (Cps2A), chain length determination (Cps2B, C), export

TABLE 3. Virulence of wild-type and capsular mutant *S. suis* strains in germfree pigs

S. suis strain[a]	No. of pigs	Mortality (%)[b]	Morbidity (%)[c]	Clinical index[d] of the group (%)		Fever index (%)[g]	Leukocyte index (%)[h]	No. of pigs in which *S. suis* was isolated from:		
				Specific symptoms[e]	Nonspecific symptoms[f]			CNS	Serosae	Joints
10	4	100	100	11	88	43	44	2	3	4
10cpsΔB	4	0	0	0	10	1	3	1	3	2
10cpsΔEF	4	0	0	0	0	1	0	1	3	2

[a] Strain 10 is the wild-type strain, and strains 10cpsΔB and 10cpsΔEF are isogenic capsular mutant strains.
[b] Percentage of piglets that died due to infection or had to be killed for animal welfare reasons.
[c] Percentage of pigs with specific symptoms.
[d] Percentage of observations which matched the described criteria.
[e] Ataxia, lameness of at least one joint, and/or stiffness.
[f] Inappetence and/or depression.
[g] Percentage of observations for the experimental group of a body temperature of >40°C.
[h] Percentage of blood samples for the group in which the concentration of granulocytes was $>10^{10}$/liter.

(Cps2C), biosynthesis (Cps2E through Cps2H, Cps2J, and Cps2K), and polymerization (Cps2I). The overall organization is similar to that of the *cps* and *eps* gene clusters of a number of gram-positive bacteria (17, 27, 32, 34). A region involved in biosynthesis is preceded by a region containing genes with more common functions. Although, based on sequence similarities, a role of most of the gene products in the polysaccharide biosynthesis could be envisaged, the role of the *orf2Z*, *orf2Y*, and *orf2X* genes remains unclear so far. The incomplete *orf2Z* gene was located at the 5′ end of the cloned fragment. Orf2Z showed some similarity to the YitS protein of *B. subtilis*. However, because the function of the YitS protein is unknown, this did not give us any information about the possible function of Orf2Z. Because the *orf2Z* gene is not a part of the *cps* operon, a role of this gene in polysaccharide biosynthesis is not expected. The Orf2Y protein showed similarity to the YcxD protein of *B. subtilis* (43). The YcxD protein was suggested to be a regulatory protein. Similarly, Orf2Y may be involved in the regulation of polysaccharide biosynthesis. The Orf2X protein showed similarity to the YAAA proteins of *H. influenzae* and *E. coli*. The function of these proteins is unknown. In *S. suis* serotype 2 the *orf2X* gene seems to be the first gene in the *cps2* operon. This suggests a role of Orf2X in polysaccharide biosynthesis. In *H. influenzae* and *E. coli*, however, these proteins are not associated with capsular gene clusters.

The products encoded by the *cps2E*, *cps2F*, *cps2G*, *cps2H*, *cps2J*, and *cps2K* genes showed similarities to glycosyltransferases of several streptococci (5, 16–18, 32). The *cps2E* gene product showed strong homology to the Cps14E protein of *S. pneumoniae* (16, 18). Cps14E is a glucosyl-1-phosphate transferase that links glucose to a lipid carrier (18). In *S. pneumoniae* this is the first step in the biosynthesis of the oligosaccharide repeating unit. The structure of the *S. suis* serotype 2 capsule is unknown, but it is composed of glucose, galactose, *N*-acetylglucosamine, rhamnose, and sialic acid in a ratio of 1:3:1:1:1 (6). Therefore, because the capsule of *S. suis* serotype 2 does contain glucose (6), we speculate that Cps2E of *S. suis* could also have glucosyltransferase activity and is probably involved in the linkage of the first sugar to the lipid carrier. The *cps2F* gene product showed homology to the Cps23fT protein, which has rhamnosyltransferase activity, of *S. pneumoniae* (5). Because rhamnose is a component of the *S. suis* serotype 2 polysaccharide (6) Cps2F could have rhamnosyltransferase activity. The *cps2G* gene encoded a protein that showed moderate similarity to the *epsF* gene product of *Streptococcus thermophilus* (32). On the basis of homology *epsF* is suggested to encode galactosyltransferase activity. Hence, a similar galactosyltransferase activity is proposed for Cps2G. The *cps2H* gene encodes a protein with an N-terminal region that is similar to the N-terminal region of the RGPEC protein of *Streptococcus mutans* (D1033055). For this protein a glycosyltransferase activity was suggested. Moreover, the hydrophobicity plots of Cps2H and RGPEC looked very similar in these regions (data not shown). Therefore, Cps2H could have glycosyltransferase activity as well. Cps2J and Cps2K showed homology to Cps14J of *S. pneumoniae* (17). Cps2J also showed homology to Cps14I of *S. pneumoniae*. Cps14I has *N*-acetylglucosaminyltransferase activity, whereas Cps14J possesses a β-1,4-galactosyltransferase activity (17). In *S. pneumoniae* Cps14I is responsible for the addition of the third sugar and Cps14J is responsible for the addition of the last sugar in the synthesis of the type 14 repeating unit (17). Because the capsule of *S. suis* serotype 2 contains galactose as well as *N*-acetylglucosamine components, galactosyltransferase *N*-acetylglucoaminyltransferase activities could be envisaged for the *cps2J* and *cps2K* gene products, respectively. The two conserved regions, DXS and DXDD, which are conserved in several glycosyltransferases (17) and which are proposed to be important for catalytic activity, were also found in Cps2J and Cps2K. The Cps2I protein showed similarity to the Cps23fI protein of *S. pneumoniae*, which has a capsular polysaccharide polymerase activity (5), suggesting that Cps2I could be involved in the polymerization of the type 2 specific oligosaccharides.

The capsule of *S. suis* serotype 2 is composed of glucose, galactose, *N*-acetylglucosamine, rhamnose, and sialic acid (6). Based on sequence homology genes encoding potential glucosyl-, galactosyl-, *N*-acetylglucosaminyl-, and rhamnosyltransferase activities could be identified. However, we have not found genes homologous to genes involved in the synthesis, activation, and transfer of sialic acid. Moreover, since we do not know whether the *cps2K* gene is the last gene in the *cps2* locus, these genes can be located downstream of *cps2K*. Therefore, in future experiments we will concentrate on the cloning and characterization of these genes. Moreover, the analysis of isogenic mutants in which the individual genes are interrupted, without disturbing expression of the downstream genes, will give more information about the role of the individual *cps2* genes in the polysaccharide biosynthesis of the *S. suis* serotype 2 capsule.

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